International Standard 5504

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Oilseeds and oilseed residues — Determination of isothiocyanates and vinyl thiooxazolidone

Graines oléagineuses et tourteaux — Dosage des isothiocyanates et de la vinylthiooxazolidone

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Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council.

International Standard ISO 5504 was developed by Technical Committee ISO/TC 34, *Agricultural food products*, and was circulated to the member bodies in January 1982.

It has been approved by the member bodies of the following countries :

Brazil	Hungary	Romania
Chile	India	South Africa, Rep. of
Czechoslovakia	Ireland	Turkey
Egypt, Arab Rep. of	Israel	United Kingdom
Ethiopia	Kenya	USSR
France	New Zealand	Yugoslavia
Germany, F. R.	Poland	

The member bodies of the following countries expressed disapproval of the document on technical grounds :

Canada
Netherlands

This International Standard has also been approved by the International Union of Pure and Applied Chemistry (IUPAC).

Printed in Switzerland

Oilseeds and oilseed residues — Determination of isothiocyanates and vinyl thiooxazolidone

1 Scope and field of application

This International Standard specifies a method for the determination of the isothiocyanates (ITC) and 5-vinyl thiooxazolidone (VTO) produced by the enzymic hydrolysis of glucosinolates in oilseeds and oilseed residues of colza (*Brassica napus*) and rapeseed (*Brassica rapa*).

Under the operating conditions described, the method does not allow determination of free isothiocyanates and free vinyl thiooxazolidone.

It also gives, in an annex, a method of determining isothiocyanates by argentimetry which may be substituted for the gas chromatographic method, by agreement between the parties. This method is not applicable, however, to the seeds or residues of colza or rapeseed having low glucosinolates contents.

2 References

ISO 659, *Oilseeds — Determination of hexane extract (or light petroleum extract), called "oil content".*

ISO 664, *Oilseeds — Reduction of contract samples to analysis samples.*

ISO 734, *Oilseed residues — Determination of hexane extract (or light petroleum extract), called "oil content".*

3 Principle

After removal of the fats and oil (de-fatting) if necessary, and drying of the oilseed or oilseed residue, enzymatic hydrolysis of the glucosinolates, extraction of isothiocyanates with dichloromethane and of vinyl thiooxazolidone with diethyl ether.

Determination of the ITC by gas chromatography and of the VTO by ultraviolet spectrophotometry.

4 Reagents

The reagents shall be of recognized analytical quality and the water used shall be distilled water or water of at least equivalent purity.

4.1 Reagents for determination of ITC

4.1.1 Dichloromethane, or, failing this, **chloroform.**

4.1.2 *n*-**Hexane,** or, failing this, **light petroleum** (boiling range 40 to 60 °C).

4.1.3 Buffer solution, of pH 7, commercially available, or, for example, a solution prepared as follows :

Measure 35,3 ml of 0,1 mol/l citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) solution (21,01 g/l solution) into a 200 ml one-mark volumetric flask and dilute to the mark with a 0,2 mol/l solution of disodium hydrogenorthophosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) (35,61 g/l solution). Check the pH and, if necessary, adjust.

4.1.4 Enzyme source, prepared from white mustard seeds (*Sinapis alba* L).[1)]

Finely grind the white mustard seeds so that at least 80 % will pass through a sieve of aperture size 280 µm.

Remove the oil and fat from the grindings using **cold** hexane or, failing this, **cold** light petroleum (4.1.2), carrying out the extraction using a method which will permit extraction until not more than 2 % of oil remains in the product and without the temperature exceeding 30 °C, for example a double-walled Soxhlet apparatus, or by carrying out extractions by grinding with hexane in a microgrinder cooled by running water. Remove traces of solvent at ambient temperature, preferably using a slight current of air.

Store the enzyme source thus obtained at 4 °C in a hermetically sealed glass bottle. In these conditions, it can be kept for about

1) Use seed in which more than 85 % of the grains germinates in less than 72 h and which is less than 2 years old.

6 weeks, but it is preferable to use a freshly prepared enzyme source.

It is recommended that a blank test be performed to ensure that the enzyme source does not contain ITC in quantities which could significantly affect the results.

4.1.5 Butyl isothiocyanate, standard solution.

Prepare a 0,500 g/l solution of butyl isothiocyanate in the dichloromethane or chloroform (4.1.1). Store at 4 °C.

This solution may be diluted if the isothiocyanates content of the sample is expected to be low.

4.1.6 Gases for gas chromatography.

Carrier gas : carefully dried nitrogen containing less than 10 mg of oxygen per kilogram.

Auxiliary gases : hydrogen and air.

4.2 Reagents for the determination of VTO

4.2.1 Diethyl ether, spectrophotometric quality.

4.2.2 Anti-foaming agent, for example octan-2-ol.

4.2.3 Buffer solution, of pH 7 (see 4.1.3).

4.2.4 Enzyme source (see 4.1.4).

5 Apparatus

Usual laboratory equipment and in particular

5.1 Apparatus for preparing the test sample and drying the test portion

5.1.1 Sieve, of aperture size 280 µm.

5.1.2 Apparatus for the extraction of fat and oil by the method specified in ISO 659 or ISO 734, if necessary.

5.1.3 Grinder.

5.1.4 Desiccator.

5.1.5 Analytical balance.

5.1.6 Electric oven, capable of being controlled at 103 ± 2 °C.

5.1.7 Conical flask, of capacity 25 ml.

5.2 Apparatus for the determination of ITC

5.2.1 Chromatograph, with a flame ionization detector and a recorder, comprising, for example, a column of length 2 m, external diameter 3,2 mm and internal diameter 2 mm, filled with 10 % diethylene glycol succinate (DEGS) on GAS/CHROM P 150 to 180 µm (80 to 100 mesh) (treated with hexamethyldisilazane).

5.2.2 Stirrer/shaker system, for conical flasks, or a **magnetic stirrer.**

5.2.3 Microsyringe, of capacity 1 µl.

5.2.4 Pipettes, of capacities 5 and 10 ml.

5.3 Apparatus for the determination of VTO

5.3.1 Spectrophotometer, preferably with a recorder, suitable for measurements in the ultraviolet, with silica cells of optical path length 10 mm.

5.3.2 Stirrer/shaker system, for conical flasks, or a **magnetic stirrer.**

5.3.3 Conical flasks, of capacities 100 and 200 ml.

5.3.4 One-mark volumetric flasks, of capacities 25 and 100 ml.

5.3.5 Beaker, of capacity 50 ml.

5.3.6 Separating funnel, of capacity 50 ml.

5.3.7 Pipette, of capacity 1 ml.

6 Procedure

6.1 Preparation of the test sample

6.1.1 Oilseeds

After reducing the laboratory sample in accordance with ISO 664, take about 10 g of the oilseed and de-fat it using the procedure specified in ISO 659. After removing the solvent, grind if necessary, so that at least 80 % of the grindings obtained pass through the sieve (5.1.1).

6.1.2 Oilseed residues

As de-fatting is not necessary for oil contents below 5 %, use the oilseed residues as received after grinding, if necessary, so that at least 80 % of the grindings obtained pass through the sieve (5.1.1).

As results are expressed relative to the de-fatted sample, it is necessary to perform a parallel determination of the oil content of the oilseed residue, using the method described in ISO 734 or any other suitable method.

However, if the oil content is greater than 5 % (pressed oilseed residues), carry out de-fatting of 10 g of oilseed residue using the procedure specified in ISO 734. After elimination of the solvent, grind, if necessary.

6.2 Determination of ITC

6.2.1 Test portion

Transfer approximately 2,2 g of the test sample (6.1) to a 25 ml conical flask (5.1.7), which has been previously dried and weighed to the nearest 1 mg. Place in the oven (5.1.6), controlled at 103 ± 2 °C, for at least 8 h, and allow to cool in the desiccator (5.1.4) to room temperature.

NOTE — Simultaneously, dry the test portion to be used for the determination of VTO in the beaker if this analysis has been requested (see 6.3.1).

Weigh the conical flask to the nearest 1 mg, and determine the mass of the de-fatted, if necessary, and dried test portion (about 2 g).

6.2.2 Determination

Add to the test portion, 5 ml of the buffer solution (4.1.3), 0,25 g of the enzyme source (4.1.4) and 10 ml, using the pipette (5.2.4), of the standard butyl isothiocyanate solution (4.1.5) (using a more dilute solution, if necessary), such that the height of the peak of the standard solution is of the same order as that of the highest peak.

Shake for 2 h at room temperature using the stirrer/shaker system or the magnetic stirrer (5.2.2). Allow to separate, or centrifuge if necessary.

Using the microsyringe (5.2.3), take 1 µl of the dichloromethane or chloroform phase, avoiding taking any particles in suspension, and inject this into the chromatograph (5.2.1) controlled, for example, as follows :

temperature of the injector : 135 °C

temperature of the column : 90 °C

temperature of the detector : 130 °C

pressure or flow rate of the carrier gas : 80 kPa* or flow rate of 20 to 30 ml/min.

The order of elution is as follows :

allyl isothiocyanate,

butyl isothiocyanate,

butenyl isothiocyanate,

pentenyl isothiocyanate.

NOTES

1 For information, if using diethylene glycol succinate (DEGS) as the stationary phase, the retention times relative to butyl isothiocyanate are as follows :

allyl isothiocyanate :	0,70
butenyl isothiocyanate :	1,45
pentenyl isothiocyanate :	2,45.

2 As the solution injected is very corrosive, clean the microsyringue immediately after use with a solvent.

6.3 Determination of VTO

6.3.1 Test portion

Transfer approximately 2,2 g of the test sample (6.1) to a 50 ml beaker (5.3.5), which has been previously dried and weighed to the nearest 1 mg. Place in the oven (5.1.6), controlled at 103 ± 2 °C, for at least 8 h, and allow to cool in the desiccator (5.1.4) to room temperature.

Weigh the beaker to the nearest 1 mg, and determine the mass of the de-fatted, if necessary, and dried test portion (about 2 g).

6.3.2 Determination

Quantitatively transfer the test portion into a 200 ml conical flask (5.3.3) and add 70 ml of boiling buffer solution (4.2.3), using some to rinse the beaker. Allow to cool to about 30 °C, then add 0,50 g of the enzyme source (4.2.4) and a few drops of the anti-foaming agent (4.2.2). Shake for 2 h at room temperature using the stirrer/shaker system or the magnetic stirrer (5.3.2).

Immediately transfer quantitatively to a 100 ml one-mark volumetric flask (5.3.4), rinsing with water, and dilute to the mark with water. Filter and collect the filtrate in a 100 ml conical flask (5.3.3). Shake gently for 30 s and, by means of the pipette (5.3.7), take 1 ml and place it in the 50 ml separating funnel (5.3.6). Carry out two extractions of the VTO using 10 ml of the diethyl ether (4.2.1) each time. Collect the ether layers in a 25 ml one-mark volumetric flask (5.3.4), and dilute to the mark with diethyl ether.

Determine the absorption curve from 220 to 280 nm and subtract the absorbance read at 280 nm from that read at the maximum absorbance, which occurs at about 250 nm, in order to obtain the absorbance of the test portion.

If the absorbance values obtained are beyond the limits of the instrument, dilute as necessary with diethyl ether.

6.3.3 Blank test

Carry out a blank test under the same conditions, omitting the test portion in order to determine the absorbance due to the enzyme source.

* 80 kPa = 0,8 bar

7 Expression of results

NOTE — For animal feeding stuffs, the results are generally expressed relative to the product as received. Thus, if such a manner of expression of results is required, make the necessary corrections to take into account the water and oil contents.

7.1 ITC content

The ITC content, expressed in milligrams per gram of dry matter of the de-fatted[1] sample, is equal to

$$\frac{m_e}{115{,}19 \times S_e \times m}\left[(4/3 \times 99{,}15 \times S_a) + (4/4 \times 113{,}18 \times S_b) + (4/5 \times 127{,}21 \times S_p)\right]$$

$$= \frac{m_e}{S_e \times m}(1{,}15\,S_a + 0{,}98\,S_b + 0{,}88\,S_p)$$

where

m is the mass, in grams, of the de-fatted and dried test portion (6.2.1);

m_e is the mass, in milligrams, of butyl isothiocyanate contained in 10 ml of solution (usually 5 mg);

S_e is the area of the peak corresponding to butyl isothiocyanate;

S_a is the area of the peak corresponding to allyl isothiocyanate;

S_b is the area of the peak corresponding to butenyl isothiocyanate;

S_p is the area of the peak corresponding to pentenyl isothiocyanate.

NOTE — The values 4/3, 4/4 and 4/5 are the theoretical response coefficients of isothiocyanates.

7.2 VTO content

The VTO content, expressed in milligrams per gram of dry matter of the de-fatted sample, is equal to

$$(A_E - A_B)\,C_p \times 25 \times 100 \times \frac{1}{m} \times 10^{-3}$$

where

A_E is the absorbance of the test solution (6.3.2);

A_B is the absorbance of the blank test solution (6.3.3);

C_p is a conversion factor, in milligrams per litre per absorbance unit, equal to 8,20;

m is the mass, in grams, of the test portion (6.3.1).

A simplified formula may thus be derived

$$\frac{20{,}5 \times (A_E - A_B)}{m}$$

Take into account any dilution of the test solution.

8 Test report

The test report shall show the method used and the results obtained. It shall also mention any operating details not specified in this International Standard, or regarded as optional, together with any incidents likely to have affected the results.

The test report shall give all the details required for the complete identification of the sample.

1) In the case of oilseed residues which have not been de-fatted, it is necessary to take into account the oil content determined in 6.1.2.

Annex

Determination of isothiocyanates by argentimetry

A.0 Introduction

For laboratories which do not have gas chromatographic apparatus for determining ITC, it is recommended to use the following argentimetric method, which was compared with the gas chromatographic method when inter-laboratory tests were carried out at the international level, and the results of which proved comparable, provided that the glucosinolates content was sufficiently high.

It is important to note, however, that the expression of results is different in the two methods; for gas chromatography, it is expressed in milligrams of isothiocyanates detected, whilst, for argentimetry, it is expressed conventionally in milligrams of butenyl isothiocyanate.

A.1 Principle

After de-fatting (if necessary) and drying of the oilseed or oilseed residue, enzymatic hydrolysis of glucosinolates, then azeotropic distillation of ITC in the presence of ethanol and collection in dilute ammonia solution. Action of silver nitrate on the thiourea derivatives thus formed.

Back titration of the excess silver nitrate using an ammonium thiocyanate solution.

A.2 Reagents and materials

The reagents shall be of recognized analytical quality and the water used shall be distilled water or water of at least equivalent purity.

A.2.1 Ethanol, 95 % (V/V).

A.2.2 Anti-foaming agent, for example octan-2-ol.

A.2.3 Nitric acid solution, $c(HNO_3)$ = 6 mol/l.

A.2.4 Ammonia, 10 % (m/m) solution.

A.2.5 Iron(III) ammonium sulphate, 80 g/l solution.

A.2.6 Silver nitrate, standard volumetric solution, $c(AgNO_3)$ = 0,1 mol/l.

A.2.7 Ammonium thiocyanate, standard volumetric solution, $c(NH_4SCN)$ = 0,01 mol/l.

A.2.8 Buffer solution, of pH 4, commercially available, or, for example, a solution prepared as follows :

Dissolve 42 g of citric acid monohydrate in 1 l of water.

Adjust to pH 4 using concentrated sodium hydroxide solution.

A.2.9 Enzyme source, prepared using white mustard seed (*Sinapis alba* L.) (see 4.1.4).

A.2.10 Anti-bumping granules.

A.3 Apparatus

Usual laboratory equipment and in particular

A.3.1 Apparatus for preparation of the test sample

See 5.1.

A.3.2 Apparatus for determination of ITC

A.3.2.1 Electric oven, capable of being controlled at 25 or 40 °C.

A.3.2.2 Distillation apparatus, of the type shown in the figure, comprising in particular :

— a conical flask (a), of capacity 500 ml;

— a round-bottomed flask (b), of capacity 250 ml, with a graduation line corresponding to a volume of 70 ml.

A.3.2.3 Ice-water bath.

A.3.2.4 Boiling water bath.

A.3.2.5 Conical flask, of capacity 100 ml.

A.3.2.6 One-mark volumetric flask, of capacity 100 ml.

A.3.2.7 Pipettes, of capacities 10 and 25 ml.

A.3.2.8 Filter paper.

A.3.2.9 Reflux condenser, to fit the flask (b) of the distillation apparatus (A.3.2.2).

A.4 Procedure

A.4.1 Preparation of the test sample

Prepare the test sample as described in 6.1.

A.4.2 Test portion

Transfer approximately 2,2 g of the test sample (A.4.1) to the conical flask, which has been previously dried and weighed to the nearest 1 mg. Place in the oven (5.1.6), controlled at 103 ± 2 °C, for at least 8 h, and allow to cool in the desiccator (5.1.4) to room temperature.

Weigh the conical flask to the nearest 1 mg, and determine the mass of the de-fatted, if necessary, and dried test portion (about 2 g).

A.4.3 Hydrolysis

Quantitatively transfer the test portion to the 500 ml conical flask (a) of the distillation apparatus (A.3.2.2), add 100 ml of the pH 4 buffer solution (A.2.8), using same to rinse the flask containing the test portion, and add 0,50 g of the enzyme source (A.2.9). Stopper the flask and shake gently, then place in the oven (A.3.2.1) for 16 h at 25 °C, or 3 h at 40 °C. During this period, shake the flask at regular intervals.

A.4.4 Preparation of the receiver flask

Transfer to the round-bottomed flask (b) of the distillation apparatus (A.3.2.2), 10 ml of the silver nitrate solution (A.2.6), measured from a pipette (A.3.2.7), and 2,5 ml of the ammonia solution (A.2.4).

Connect the flask to the distillation apparatus and place it in the ice-water bath (A.3.2.3). The end of the condenser tube shall be immersed in the silver nitrate-ammonia solution.

A.4.5 Distillation

Allow the conical flask (see A.4.3) to cool to room temperature, add a few anti-bumping granules (A.2.10) and a few drops of the anti-foaming agent (A.2.2), then connect it to the distillation apparatus. Using a funnel placed above the condenser, add to the contents of the flask 10 ml of the ethanol (A.2.1) and place 3 ml of the ethanol in the safety tube of the receiver flask.

Distil slowly until a total of 70 ml of liquid is obtained in the receiver flask.

A.4.6 Determination

Disconnect the receiver flask, and pour into it the ethanol contained in the safety tube. Attach a reflux condenser and heat the flask for 30 min on a boiling water bath, then cool in cold water.

Transfer quantitatively to a 100 ml volumetric flask (A.3.2.6), rinsing the flask with water, and dilute to the mark. Shake, and filter on a filter paper. Using a pipette (A.3.2.7), take 25 ml of the filtrate and place it in a 100 ml conical flask (A.3.2.5). Add 1 ml of the nitric acid solution (A.2.3), and 0,5 ml of the iron(III) ammonium sulphate solution (A.2.5) as indicator.

Titrate the excess silver nitrate with the ammonium thiocyanate solution (A.2.7) until a stable pink colour is obtained.

A.4.7 Blank test

Carry out a blank test using the same procedure, but omitting the test portion.

A.5 Expression of results

The ITC content, expressed as butenyl isothiocyanate in milligrams per gram of dry matter of the de-fatted[1] sample, is equal to

$$\frac{4\,(V_1 - V_2) \times c \times 56{,}59}{m}$$

where

m is the mass, in grams, of the test portion (A.4.2);

V_1 is the volume, in millilitres, of ammonium thiocyanate solution used for the blank test (A.4.7);

V_2 is the volume, in millilitres, of ammonium thiocyanate solution used for the determination (A.4.6);

c is the exact concentration of the ammonium thiocyanate solution used.

A.6 Test report

The test report shall show the method used and the results obtained. It shall also mention any operating details not specified in this International Standard, or regarded as optional, together with any incidents likely to have affected the results.

The test report shall give all the details required for the complete identification of the sample.

1) In the case of oilseed residues which have not been de-fatted, it is necessary to take into account the oil content determined in 6.1.2.

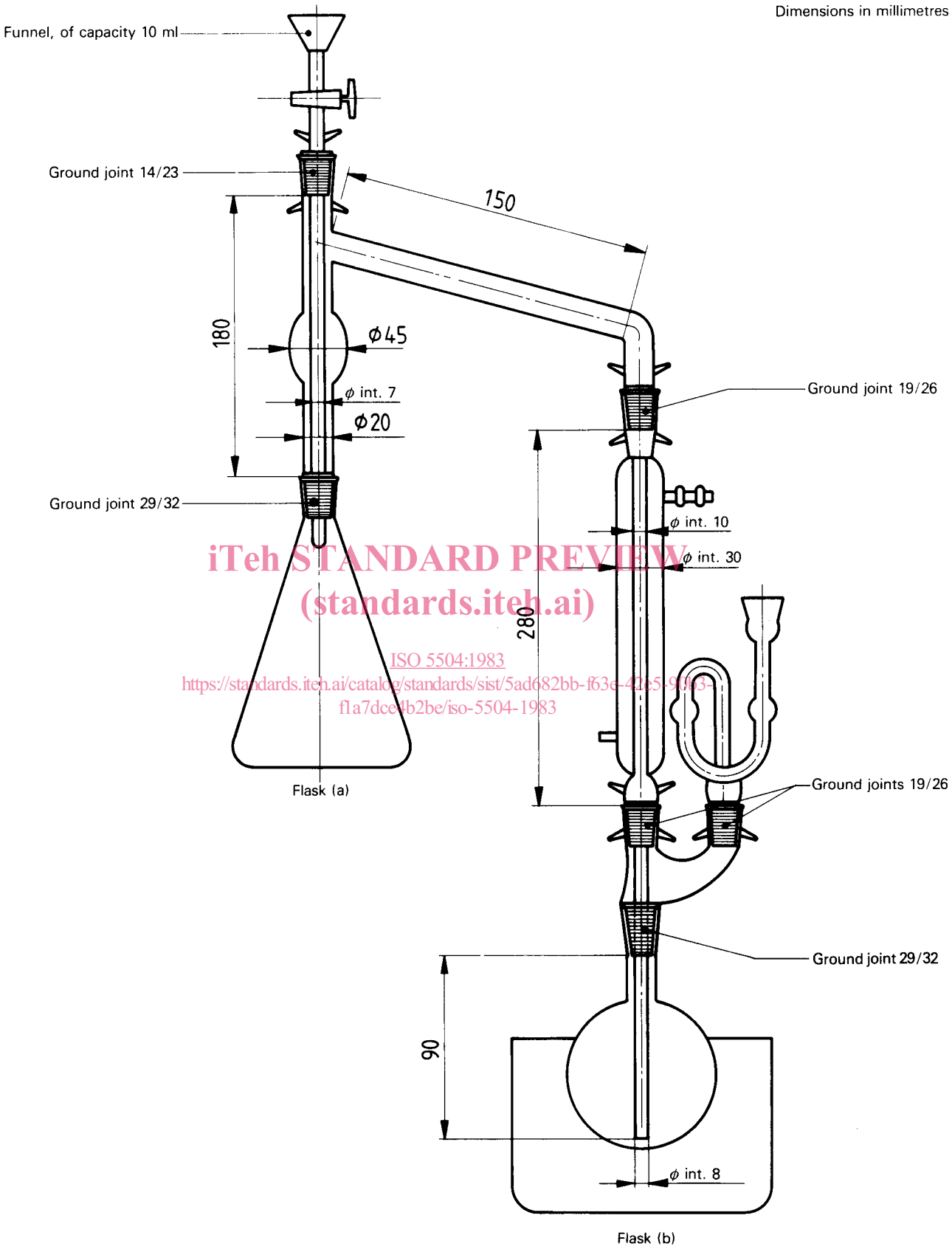

Figure — Example of apparatus for the distillation of isothiocyanates